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14. ABSTRACT

The research goals of this grant proposal are to: 1) investigate the effect of ETS gene fusions on radiation phenotype in preclinical models of prostate cancer, 2) to explore the mechanism of interaction between ERG (the predominant ETS gene fusion product) and the DNA repair protein DNA-PK, and 3) to determine if ETS gene fusion status is a clinical biomarker of radioresistance for prostate cancer. The training goals of this grant proposal included a series of regular meetings with mentors, research seminars, journal clubs, and workshops, all of which are intended to help Dr. Feng develop as a translational scientist. This grant proposal was approved as a five-year award; the current annual report summarizes accomplishments over the first year of the grant, from July 15, 2010 to July 15, 2011.

Overall, the first year of this grant period has been very successful. The work accomplished as a result of this grant has let to a publication in a very high-impact journal (*Cancer Cell)*, three national presentations, and a funded Young Investigator Award from the Prostate Cancer Foundation. Additionally, Dr. Feng has met the training achievements specified in his original grant.

The research proposed in this training grant represents an important area within the field of prostate cancer research. Because ETS gene fusions are thought to be driver alterations in over half of all prostate cancers, understanding the mechanistic and potential clinical implications of these gene fusions has significant ramifications, particularly in the context of radiation therapy, which represents one of the primary treatment modalities for localized prostate cancer. Our first-year findings are that ERG (the predominant ETS gene fusion product) confers radiation resistance in preclinical models of prostate cancer and that this radiation resistance can be reversed with DNAPK inhibition. These findings suggest that DNA-PK inhibition should be explored as a clinical strategy for radiosensitizing prostate cancers. In addition, we have discovered that ERG interacts with DNA-PK via its Y373 residue, suggesting a future approach that can be developed to more specifically radiosensitize ERG-positive cancers.

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Introduction

This annual report will summarize the accomplishments associated with the Department of Defense Physician Research Training Award (W81XWH-10-1-0582), awarded to Felix Feng, M.D. This award included both research goals and training goals. The research goals of this grant proposal are to: 1) investigate the effect of ETS gene fusions on radiation phenotype in preclinical models of prostate cancer, 2) to explore the mechanism of interaction between ERG (the predominant ETS gene fusion product) and the DNA repair protein DNA-PK, and 3) to determine if ETS gene fusion status is a clinical biomarker of radioresistance for prostate cancer. The training goals of this grant proposal included a series of regular meetings with mentors, research seminars, journal clubs, and workshops, all of which are intended to help Dr. Feng develop as a translational scientist, with the ultimate goals of submitting a NIH-level grant as an independent investigator and developing a translational clinical trial. This grant proposal was approved as a five-year award; the current annual report summarizes accomplishments over the first year of the grant, from July 15, 2010 to July 15, 2011.

Body

Research achievements: Tasks and Subtasks

As outlined in the original Statement of Work, this grant proposal was comprised of three specific aims, subdivided into 7 tasks, which were further divided into 20 subtasks. The original goals for year #1 of the grant were to accomplish six subtasks (1A, 1B, 2A, 2B, 3A, and 4A), resulting in full completion of Task #1. In actuality, I was able to exceed these proposed goals, and was able complete seven subtasks (1A, 1B, 3A, 3B, 4A, 4B, and 4C), resulting in completion of Tasks #1 and #3. The findings associated with these subtasks and tasks are detailed below.

Task #1 was to determine the impact of ERG overexpression on *in vitro* cell survival following radiation (+/- DNAPK inhibition and/or androgen ablation). Specifically, subtask 1A was to develop models of ERG overexpression and subtask 1B was to perform clonogenic survival assays using these models. Figures 1A, 1B, and 1C (see Figures section) show the results of these experiments. We stably overexpressed ERG in both PC3 and DU145 prostate cancer cell lines, and then assessed clonogenic survival in these cell lines, following radiation alone or in combination with DNA-PK knockdown or inhibition. Specifically, in Figure 1A, we used siRNA approaches to knockdown DNA-PK; in Figure 1B, we used the preclinical drug NU7026 to inhibit DNA-PK; in Figure 1C, we used the preclinical drug NU7441 to inhibit DNA-PK. All of these figures show similar findings:

a) overexpression of ERG results in radiation resistance in clonogenic survival assays
b) knockdown or inhibition of DNAPK reverses the radiation resistance conferred by ERG, and preferentially radiosensitizes ERG-positive cells compared to ERG-negative cells

In addition to overexpressing ERG in prostate cancer cell lines which did not express ERG at baseline, we also attempted to knockdown ERG in the single prostate cancer cell line (VCaP) which endogenously harbors the TMPRSS2:ERG gene fusion (and thus endogenously overexpresses ERG). However, we found that stable knockdown of ERG in VCaP cells massively impaired the viability of these cells at baseline, making clonogenic survival experiments with these cell lines not practical. As VCaP cells is the only androgen-sensitive prostate cancer cell line that we have access to, we could not assess the effect of androgen ablation on ERG-mediated radioresistance in vitro, but will plan on doing this with in vivo xenograft experiments (as opposed to in vitro clonogenic survival studies).

Task #3 was to evaluate the effect of ERG overexpression/knockdown on the extent and time course of DNA repair following radiation, using standard assays assessing DNA damage or repair, such as assessment of gamma H2A.X foci (subtask 3A) and COMET tail moments (subtask 3B). In particular, using COMET assays (subtask 3B), we discovered that ERG-overexpressing cells repair double-stranded DNA damage more quickly following radiation therapy in both PC3 (Figure 2A) and DU145 prostate cancer cells (Figure 2B), but that this DNA repair advantage is reversed with the addition of the DNAPK inhibitor NU7026 to radiation. In fact, the combination of radiation and DNAPK inhibition induced significantly more DNA damage in ERG-positive cells, compared to ERG-negative cells, at longer time points following therapy (Figures 2A and 2B). By assessing gamma H2A.X foci (subtask 3A), we were able to confirm the finding that ERG-overexpressing cells have more efficient repair of DNA damage following radiation alone. However, during the course of these experiments, we realized that gamma H2A.X may itself be a target of DNA-PK itself[1], meaning that measurement of gamma

H2A.X foci with immunofluorescence analysis, as initially proposed in our grant, may not represent a reliable assay for measuring DNA damage in the context of DNAPK inhibition. Therefore, we chose to focus on COMET assays, instead of gamma H2A.X foci studies, for subsequent experiments assessing DNA damage.

Task #4 was to determine which regions within the ERG protein are responsible for interacting with DNAPK and which phenotypes are mediated by this interaction. Specifically, subtask 4A was to create FLAG-tagged ERG mutants, consisting of serial 20-30 amino acid deletions in the carboxyl half of ERG, using PCR techniques. Subtask 4B was to perform immunoprecipitation (with a FLAG antibody) using lysates from cells transiently transfected with the mutants from 4A, and to probe these immunoprecipitates for DNAPK. Subtask 4C was to stably overexpress the ERG mutants which do not interact with DNAPK in ERG-negative prostate cell lines. Subtask 4D was to determine which ERG-mediated phenotypes, such as radioresistance (in clonogenic survival assays) or invasion (in Boyden chamber assays), are present in cells overexpressing wild-type ERG versus those overexpressing these mutants (from subtask 4C). *Over the first year of this grant, we were able to complete subtasks 4A, 4B, and 4C*, as shown in Figure 3.

Figures 3A and 3B demonstrate the preliminary data that was included in our original grant submission. Figure 3A is a schematic showing a series of flag-tagged ERG expression vectors with tiling deletions; each of these deletions was approximately 100 amino acids in length, and spanned either the N terminus, the pointed (PNT) domain, the middle amino acids, the ETS domain, or the C terminus. Figure 3B demonstrates the results from immunoprecipitation experiments performed in HEK293 cells transfected with expression vectors harboring the deletion constructs shown in Figure 3A, and demonstrate that an area within the middle amino acids, the ETS domain, or the C terminus (amino acids 197-479) are responsible for the interaction between ERG and DNAPK.

Because we were concerned that the size of the tiling deletions used in Figures 3A and 3B may have affected protein folding, resulting in non-local effects on ERG-DNAPK binding, we proposed to create additional ERG mutants, consisting of smaller amino acid deletions, from amino acids 197-479 (subtask 4A). We first started by creating N-terminal HALO-tagged expression vectors for in vitro purification of these ETS fragments, as shown in the schematic from Figure 3C. Using a cell-free in vitro system, we then performed immunoprecipitation-western blot experiments (subtask 4B), which demonstrated that the domain of ERG that interacted with DNAPK was the ETS domain (amino acids 310 to 393) (Figure 3D). We then utilized a series of three smaller HALO-tagged fragments that tiled the ETS domain, which localized the interaction to the final 28 amino acids of the ETS domain (Figures 3C and 3D). We also performed these immunoprecipitation-western blot experiments in the presence or absence of ethidium bromide (EtBr), which disrupts DNA-protein interactions, and demonstrated that the ERG-DNA-PK interaction is a protein-protein interaction instead of a protein-DNA-protein interaction (Figure 3D).

Because our preliminary data had demonstrated that ERG interacts with both DNAPK and another DNA repair protein, PARP1, we then performed similar immunoprecipitation-western blot experiments using our HALO-tagged ERG fragments and PARP1, in a cell-free in vitro system (Figure 3E). This also demonstrated that ERG interacts with PARP1 via the final 28 amino acids of the ETS domain, but that the ERG-PARP1 interaction is dependent upon DNA, as opposed to the ERG-DNAPK interaction (Figure 3D).

Because the crystal structure of other ETS proteins has previously been demonstrated[2], we were able to use this information to predict 5 amino acids (from residues 372-376), within the ETS domain, which may be responsible for the ERG-DNAPK interaction. By site-directed mutagenesis of each residue to alanine, we demonstrated that the Y373A mutant was unable to precipitate DNAPK, suggesting that the ERG-DNAPK interaction is mediated by Tyrosine 373 of ERG (Figure 3F). Analysis of the ETS1 structure shows that Y373 is adjacent to the arginine residues that fit into the DNA groove and that Y373 is accessible to potential interacting proteins[2]. We have now overexpressed the ERG Y373A mutant into PC3 prostate cancer cells (subtask 4C), and are currently performing further experiments to analyze the phenotypes altered with this alteration (subtask 4D ongoing but not completed).

Subtasks 4A, 4B, and 4C clearly represented a great deal of work; in fact, we had anticipated, in our original statement of work, that it would take up to 54 months to complete these 3 subtasks. However, we were fortunate that our immunoprecipitation experiments proceeded smoothly, and have completed this

work 3 years ahead of schedule. These findings recently formed part of the foundation for a *Cancer Cell* paper that I co-published this year with my mentor and primary collaborator, Arul Chinnaiyan, in which I cited my DOD support[3]; this manuscript is attached to the current application.

I would also like to note that, in my original Statement of Work, I had planned on completing subtasks 2A and 2B in the first year of this project. Because my team was making such good progress on subtasks 4A, 4B, and 4C, I decided to focus our group's efforts on the 4A-4C subtasks instead of the 2A-2B subtasks. While I was unable to complete subtasks 2A-2B this year, my team was able to compensate for this by finishing 4A-4C much ahead of schedule, as I have noted above. In fact, subtasks 4A-4C represent a great deal more work than subtasks 2A-2B. Another issue with subtask 2A (obtaining institutional approval for proposed mouse xenograft work) is that I have been somewhat hesitant to use the DNAPK inhibitor for which I have in vivo quantities (NU7026), given the modest in vitro results seen on clonogenic survival assay (Figure 2B), particularly in comparison to another DNAPK inhibitor (NU7441, Figure 2C). I am currently trying to obtain in vivo quantities of a more potent DNA-PK inhibitor; given the high cost of these drugs (particularly in the context of amounts needed for in vivo work), I am currently trying to set up a formal collaboration with companies that have DNA-PK inhibitors in early phase clinical trials.

Research achievements: Milestones

In the original Statement of Work, 11 milestones were identified, and targeted over the 5 year course of this grant. The goal for year #1 of the grant was to complete 1 out of 11 milestones (Milestone #1). While I was unable to complete Milestone #1 due to difficulty in obtaining an effective DNA-PK inhibitor for in vivo studies (see previous paragraph), I was instead able to complete parts of Milestones #2, #4, and #5, for a total of 3 out of 11 milestones reached. Specifically, Milestone #2 included presentation of the ERG-mediated *in vitro* radiation phenotype at a national meeting; I presented these findings as a poster discussion during the 2011 ASCO Annual Meeting[4], and was subsequently invited to give an oral presentation of this work at the 2011 Prostate Cancer Foundation Annual Meeting. Milestone #4 consisted of presentation on the ERG-DNAPK interactions at a national meeting; one of the medical students working in my laboratory gave an oral presentation on this work at the 2010 ASTRO Annual Meeting[5]. Milestone #5 included publications on the ERG-DNAPK interaction; as mentioned earlier, I co-published these findings with my mentor and primary collaborator, Arul Chinnaiyan, in *Cancer Cell* earlier this year[3].

Training achievements

In my original grant application, I highlighted a series of training program activities which I hoped would contribute substantially to my scientific development. Over the past year, as proposed, I have attended a number of basic science seminars, hosted by the Departments Medicine, and Molecular and Cellular Biology, which have broadened by scientific knowledge within my field. I have also attended a grant writing workshop ("Optimizing the presentation of your NIH grant") offered by my medical center. I have also regularly attended Gene Fusion and Cancer Biology Research Meetings, run by my mentor Arul Chinnaiyan, as well as the Pathology and Radiation Oncology Research Seminars, run by the two departments with which I am affiliated. Additionally, I have renewed my "Training in the Responsible Conduct of Research" certification, and presented at the national meetings noted above in the milestones section. Finally, I have met regularly with my mentors, Drs. Arul Chinnaiyan, Ted Lawrence, and Tom Carey, as planned in my original proposal.

Figures:

Figures 1-3, which are referred to in the Body section of this report, are included in the subsequent three pages. The captions for each figure are included below:

Figure 1: ERG causes radiation resistance, which is reversed by DNA-PK inhibition or knockdown:
In both PC3 and DU145 cells, ERG overexpression resulted in a 1.3 fold increase in clonogenic survival following radiation (shown in the shift from the heavy black line with circles to the heavy black line with triangles). DNAPK knockdown or inhibition was achieved with siRNA approaches (Figure 1A), the DNAPK inhibitor NU7026 (Figure 1B), or the DNAPK inhibitor NU7441 (Figure 1C). Each of these preferentially radiosensitized ERG-positive cells compared to ERG-negative cells, with an enhancement ratio of 1.6-1.7 (for the siRNA and NU7441 approaches, Figures 1A and 1C) or an enhancement ratio of 1.2 (for the NU7026 approach, Figure 1B). Western blots in Figure 1A confirm genetic knockdown of DNAPK with siRNA approaches. Drug concentrations of DNAPK inhibition were selected based on previously reported results in the public literature.

Figure 2: ERG overexpression confers increased DNA repair efficiency, which is reversed with DNAPK inhibition: Neutral COMET assays were performed at baseline, and at various time points following 15 Gy of radiation (RT) in control and ERG-positive PC3 (Figure 2A) or DU145 (Figure 2B) prostate cancer cells. Compared to control cells, ERG overexpression resulted in significantly decreased mean tail moments at various time points (1, 10, and 30 minutes) following treatment with radiation, consistent with quicker repair of double-stranded DNA damage. This ERG-associated DNA repair advantage was reversed with DNAPK inhibition (NU7026 10 μM). Stars indicate significant differences in mean tail moment between ERG-positive and control cells. Representative mages from 30 min after treatment are shown for PC3 cells (Figure 2A).

Figure 3: ERG interacts with DNA-PK in a DNA-independent manner at its tyrosine 373 residue:
Figure 3A shows a series of flag-tagged ERG expression vectors with tiling deletions; each of these deletions is approximately 100 amino acids in length, and spanned either the N terminus, the pointed (PNT) domain, the middle amino acids, the ETS domain, or the C terminus (ΔN, ΔP, ΔM, ΔE, ΔC respectively). Figure 3B demonstrates the results from immunoprecipitation experiments performed in HEK293 cells transfected with expression vectors harboring the deletion constructs shown in Figure 3A. Specifically, the input western is shown on the left and the immunoprecipitation-western blot is shown on the right. Together, these findings demonstrate that either the middle amino acids (ΔM), the ETS domain (ΔE), or the C terminus (ΔC) (amino acids 197-479) are responsible for the interaction between ERG and DNAPK. Figure 3C shows N-terminal HALO-tagged expression vectors created for in vitro purification of these ETS fragments. Figure 3D demonstrates the results from immunoprecipitation-western blot experiments using the ETS fragments depicted in Figure 3C, using a cell-free system. Together, these findings localize the ERG-DNAPK interaction to the final 28 amino acids of the ETS domain (of the ERG protein). These immunoprecipitation-western blot experiments were performed in the presence or absence of ethidium bromide (EtBr), which disrupts DNA-protein interactions, and demonstrated that the ERG-DNA-PK interaction is a protein-protein interaction instead of a protein-DNA-protein interaction. Figure 3E shows the results from similar immunoprecipitation-western blot experiments using the HALO-tagged ERG fragments and PARP1, in a cell-free system. This also demonstrated that ERG also interacts with PARP1 via the final 28 amino acids of the ETS domain, but that the ERG-PARP1 interaction is dependent upon DNA, as opposed to the ERG-DNAPK interaction (Figure 3D). Figure 3F demonstrates the results of immunoprecipitation-western blot experiments, assessing for interaction of ERG mutants with DNAPK. The ERG mutants were created using site-directed mutagenesis of each amino acid residue from position 372-376, with substitution to alanine. The Y373A mutant was unable to precipitate DNAPK, suggesting that the ERG-DNAPK interaction is mediated by Tyrosine 373 of ERG.

Figure 1

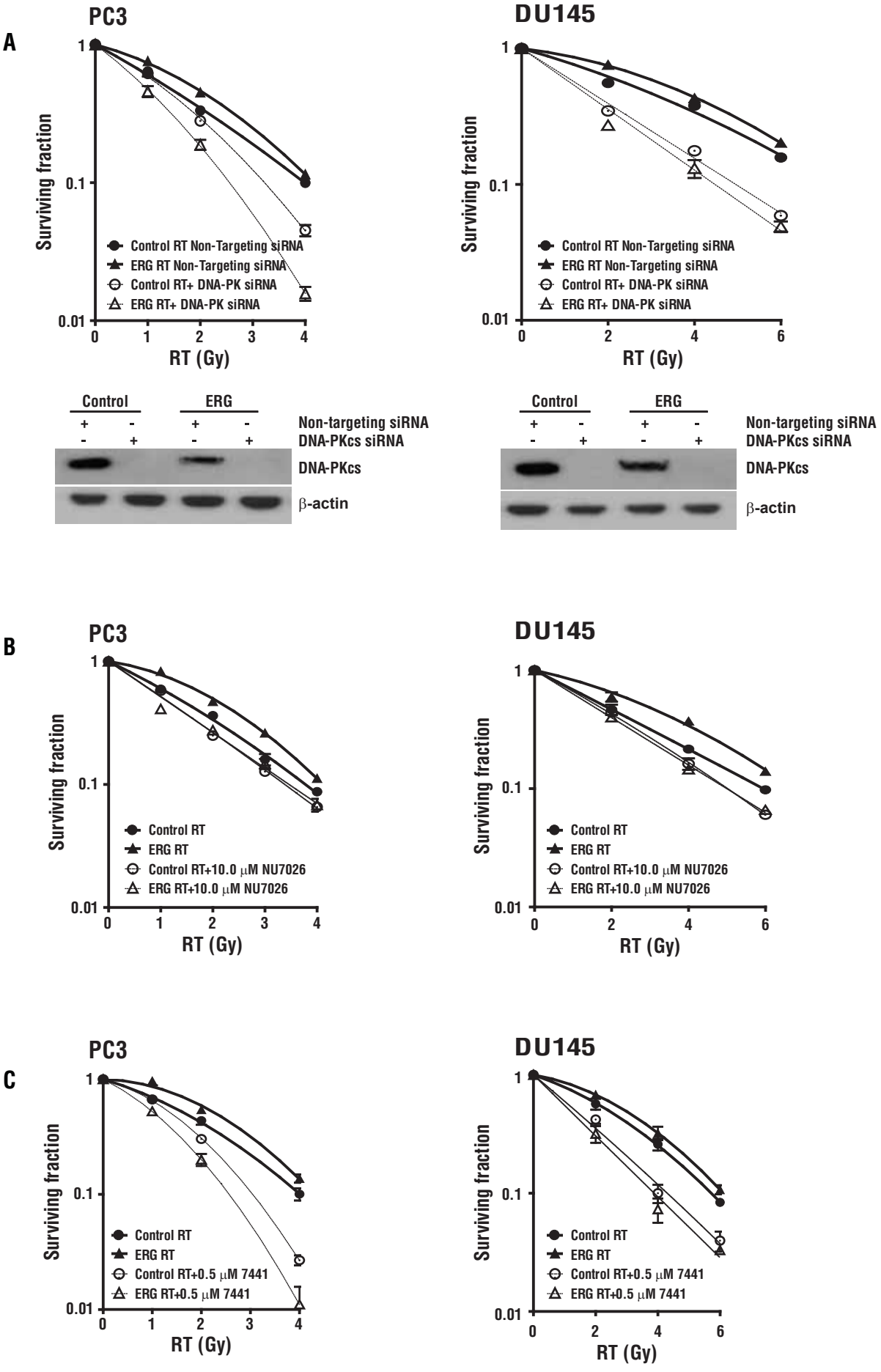
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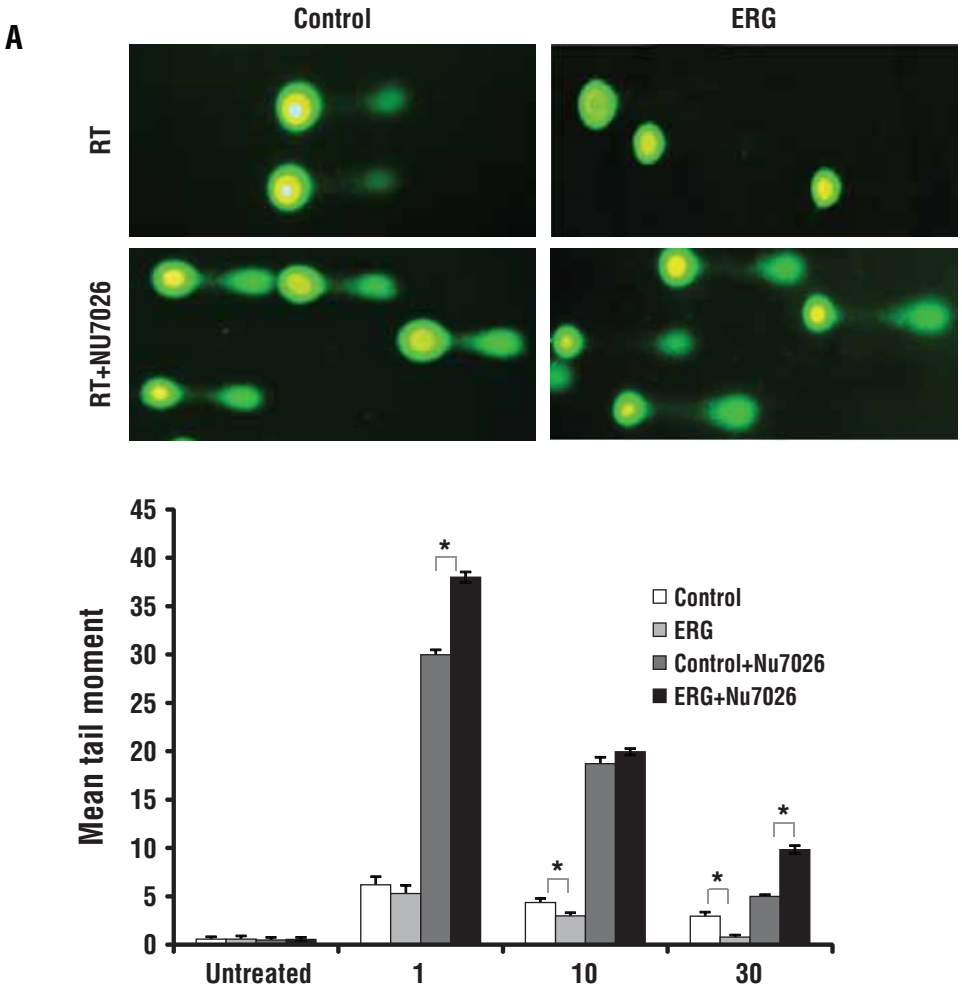
Figure 2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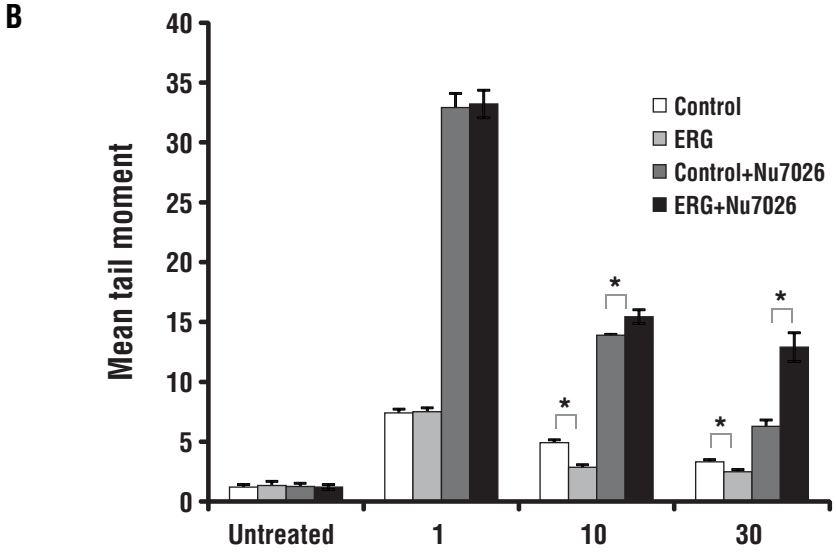
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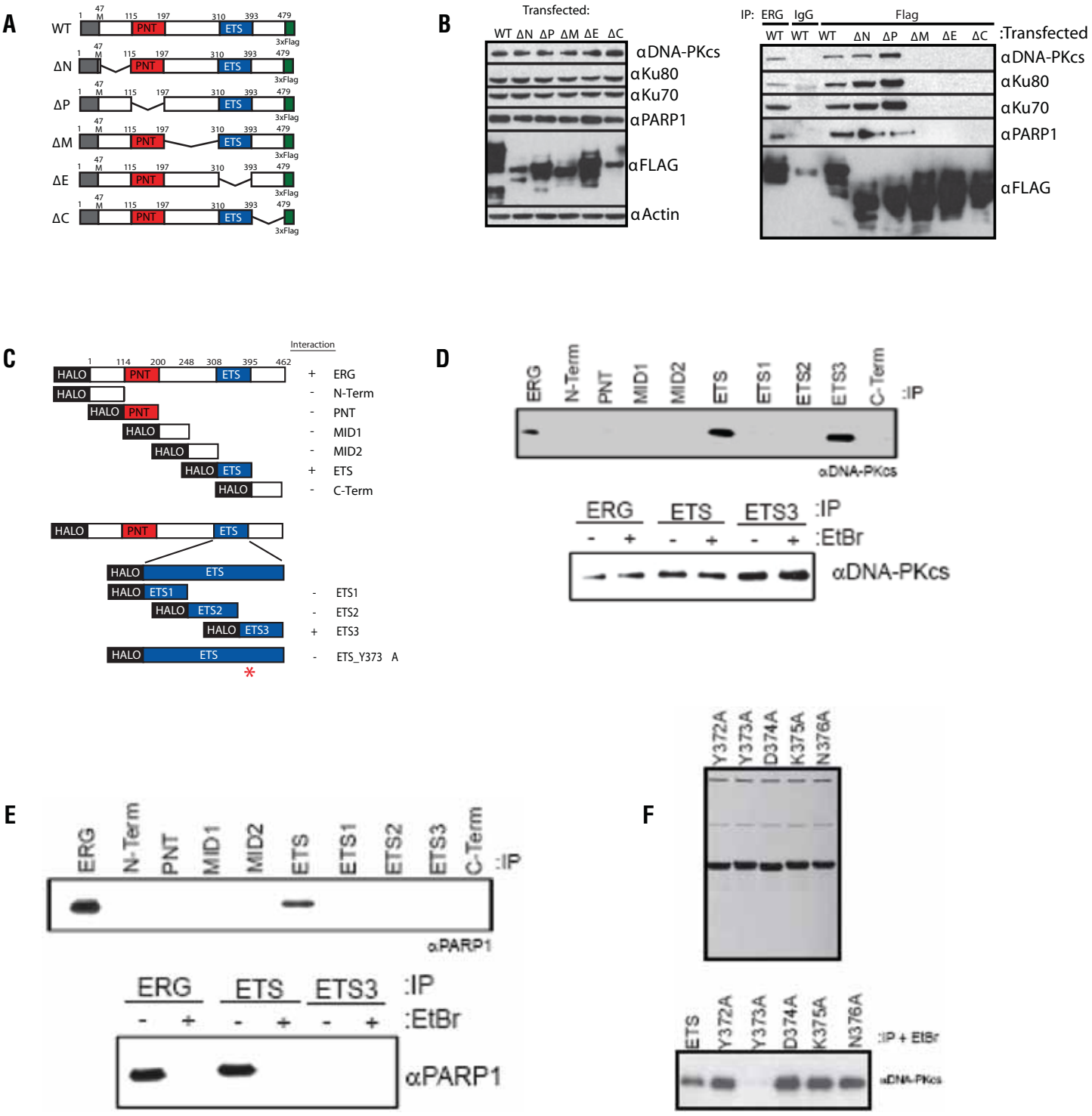
Figure 3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Key Research Accomplishments:

The key research accomplishments from the first year of this grant proposal include our findings that:

- ERG overexpression in prostate cancer cell lines confers radiation resistance
- This ERG-associated radiation resistance is mediated by increased efficiency of DNA repair in response to radiation
- ERG interacts with the repair protein DNAPK in a DNA-independent manner, at its tyrosine 373 site
- DNAPK knockdown or inhibition preferentially radiosensitizes ERG-positive vs ERG-negative cells, and can reverse ERG-mediated radiation resistance

Other key research accomplishments during this grant period include:

- Generation of PC3 and DU145 prostate cancer cell lines stably overexpressing ERG or a vector control
- Construction of ERG deletion constructs, with larger tiling deletions spanning the five domains of ERG, as well as with smaller deletions bridging the ETS domain of ERG
- Construction of an ERG mutant in which the critical Y373 residue has been mutated to alanine
- Overexpression of the above ERG mutant in PC3 prostate cancer cell lines

Reportable Outcomes:

The first year of work from this grant proposal has generated the following reportable outcomes:

1) Publication of work from Task #4 in a *Cancer Cell* manuscript[3], co-published with my mentor and primary collaborator, Dr. Arul Chinnaiyan
2) Oral presentation on work from Task #4, at the 2010 American Society of Therapeutic Radiology and Oncology Annual Meeting[5]
3) Poster discussion presenting work from Tasks #1 and #3, at the 2011 American Society of Clinical Oncology Annual Meeting[4]
4) Invited oral presentation on work from Tasks #1 and #3, at the 2011 Prostate Cancer Foundation Annual Meeting
5) A funded Young Investigator Award from the Prostate Cancer Foundation ($225,000 over 3 years), entitled "*Cooperativity between TMPRSS2:ERG Gene Fusions and PTEN Genomic Deletions in the Radiation Resistance of Prostate Cancer",* from January 2011 to January 2014

Conclusion:

This Annual Report summarizes the first-year accomplishments associated with the Department of Defense Physician Research Training Award (W81XWH-10-1-0582), awarded to Felix Feng, M.D. Overall, the first year of this grant period has been very successful. The work accomplished as a result of this grant has let to one publication in a very high-impact journal[3], three national presentations, and a funded Young Investigator Award from the Prostate Cancer Foundation. Dr. Feng has exceeded the first-year goals proposed on his original Statement of Work, and has completed 7 subtasks and 2 tasks (compared to the 6 subtasks and 1 task originally proposed). Additionally, Dr. Feng has met the training achievements specified in his original grant.

The research proposed in this training grant represents an important area within the field of prostate cancer research. Because ETS gene fusions are thought to be driver alterations in over half of all prostate cancers, understanding the mechanistic and potential clinical implications of these gene fusions has significant ramifications, particularly in the context of radiation therapy, which represents one of the primary treatment modalities for localized prostate cancer. Our first-year findings are that ERG confers radiation resistance in preclinical models of prostate cancer and that this radiation resistance can be reversed with DNAPK inhibition. These findings suggest that DNA-PK inhibition should be explored as a clinical strategy for radiosensitizing prostate cancers. In addition, we have discovered that ERG interacts with DNA-PK via its Y373 residue, suggesting a future approach that can be developed to more specifically radiosensitize ERG-positive cancers.

In terms of future work on this grant, we would like to highlight one major finding from our recent *Cancer Cell* manuscript—that PARP1 inhibition is also an effective therapy that preferentially inhibits growth of ERG-positive prostate cancer cells and xenografts compared to ERG-negative prostate cancer cells and xenografts. We have recent unpublished data (not generated as part of this grant) that PARP1 inhibition, as a single agent, may be as or more effective than DNAPK inhibition, as a single agent in preclinical models of prostate cancer (preliminary studies). Therefore, we would ask the DOD Annual Report review committee to consider allowing us to expand the aims of this grant to assess PARP1 inhibition as a therapeutic strategy for radiosensitizing ERG-positive prostate cancers. Over the next year, we would still propose to perform the same tasks specified in the original Statement of Work for this grant, but would proposed to use both DNAPK inhibitors and PARP1 inhibitors (individually) within these tasks, so that we could assess both of these therapeutic strategies. If we find that PARP1 inhibitors are consistently better than DNAPK inhibitors (in terms of radiosensitizing ERG-positive prostate cancers), we may, in the future, ask for permission to shift the majority of our work to PARP1 inhibition. Additionally, since PARP1 inhibitors are much farther along in clinical development than DNAPK inhibitors (i.e., PARP1 inhibitors are in phase III trials in other disease sites, as opposed to DNAPK inhibitors, which are only in early phase I trials), we also believe that PARP1 inhibition may represent a quicker future route to translating preclinical findings to the clinic.

I would like to thank the DOD review committee for providing us this grant to accomplish the proposed research.

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Appendices

Appendix #1: *Cancer Cell* manuscript (Reference #3)

Cancer Cell
Article

Mechanistic Rationale for Inhibition of Poly(ADP-Ribose) Polymerase in ETS Gene Fusion-Positive Prostate Cancer

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SUMMARY

Recurrent fusions of ETS genes are considered driving mutations in a diverse array of cancers, including Ewing's sarcoma, acute myeloid leukemia, and prostate cancer. We investigate the mechanisms by which ETS fusions mediate their effects, and find that the product of the predominant ETS gene fusion, *TMPRSS2:ERG*, interacts in a DNA-independent manner with the enzyme poly (ADP-ribose) polymerase 1 (PARP1) and the catalytic subunit of DNA protein kinase (DNA-PKcs). ETS gene-mediated transcription and cell invasion require PARP1 and DNA-PKcs expression and activity. Importantly, pharmacological inhibition of PARP1 inhibits ETS-positive, but not ETS-negative, prostate cancer xenograft growth. Finally, overexpression of the *TMPRSS2:ERG* fusion induces DNA damage, which is potentiated by PARP1 inhibition in a manner similar to that of BRCA1/2 deficiency.

INTRODUCTION

ETS transcription factors are aberrantly expressed in a diverse array of cancers, including prostate, breast, melanoma, and Ewing's sarcoma (Jané-Valbuena et al., 2010; Jeon et al., 1995; Shurtleff et al., 1995; Sorensen et al., 1994; Tognon et al., 2002; Tomlins et al., 2005). In prostate cancer, recurrent gene fusions of the androgen-regulated gene, *TMPRSS2*, to the oncogenic ETS transcription factor *ERG* are present in approximately 50% of prostate cancers (Brenner and Chinnaiyan, 2009; Kumar-Sinha et al., 2008; Tomlins et al., 2005). Although *ERG* is the predominant ETS gene rearrangement observed, other ETS

Significance

Although genomic rearrangements leading to ETS gene overexpression occur in about 50% of prostate cancers, transcription factors like the ETS genes have been notoriously difficult to target therapeutically. Here, we show that the ETS:PARP1 interaction axis may represent a target for therapeutic intervention in cancers with ETS gene fusions. Our study also suggests that inhibition of cofactors necessary for function may represent a promising paradigm of treatment for malignancies driven by oncogenic transcription factors. Furthermore, our study motivates the assessment of ETS gene fusions as a potential biomarker of response in future clinical trials incorporating PARP inhibitors into the treatment of prostate cancer and other ETS fusion-positive malignancies.

transcription factors are found at a much lower frequency in prostate cancer, including *ETV1* (Tomlins et al., 2005), *ETV4* (Tomlins et al., 2006), and *ETV5* (Helgeson et al., 2008). ETS gene fusions appear early in prostatic disease during the transition from high-grade prostatic intraepithelial neoplasia (PIN) lesions to invasive carcinoma (Helgeson et al., 2008; Hermans et al., 2008; Klezovitch et al., 2008; Tomlins et al., 2007a; Wang et al., 2008) and are formed by several mechanisms, including interstitial deletion and genomic insertion (Perner et al., 2007). In prostate cell lines devoid of the *TMPRSS2:ERG* gene fusion, androgen receptor-induced proximity can trigger topoisomerase-2β-mediated *TMPRSS2:ERG* gene fusion formation (Haffner et al., 2010), which is significantly enhanced by genotoxic stresses such as ionizing radiation (Lin et al., 2009; Mani et al., 2009).

Once an ETS gene fusion is formed through genomic rearrangement, the subsequent overexpression of an ETS gene fusion protein can contribute to cancer progression through several different mechanisms. For example, *TMPRSS2-ERG* gene fusion expression is required for cell growth in cell line models that harbor an endogenous gene fusion both in vitro and in vivo (Helgeson et al., 2008; Sun et al., 2008; Tomlins et al., 2007a; Wang et al., 2008). Likewise, ETS proteins are active transcription factors that drive cellular invasion through the induction of a transcriptional program highly enriched for invasion-associated genes (Helgeson et al., 2008; Hermans et al., 2008; Klezovitch et al., 2008; Tomlins et al., 2007a; Wang et al., 2008). Genetically engineered mice expressing ERG or ETV1 under androgen regulation exhibit PIN-like lesions but do not develop frank carcinoma, suggesting that additional collaborating mutations may be required for de novo carcinogenesis (Carver et al., 2009; King et al., 2009; Klezovitch et al., 2008; Kumar-Sinha et al., 2008; Tomlins et al., 2007a; Zong et al., 2009). Importantly, overexpression of ERG leads to accelerated carcinogenesis in mouse prostates with deletion of the tumor suppressor PTEN (Carver et al., 2009; King et al., 2009). Additionally, in a transplant model, mouse prostate epithelial cells (PrECs) that are forced to overexpress both ERG and the androgen receptor gene form invasive prostate cancer (Zong et al., 2009). This suggests that ERG rearrangements can function to accelerate prostate carcinogenesis.

Given the functional consequences of ETS gene rearrangements in prostate cancer progression, a critical question remains unanswered: Are ETS gene fusions therapeutic targets, either directly or indirectly? Given the difficulties in targeting nuclear transcription factors using conventional therapeutic strategies (Darnell, 2002), we hypothesized that associated enzymes critical for ERG function may instead serve as viable therapeutic targets to inhibit ETS-positive prostate cancer cell growth.

RESULTS

Identification of ERG-Interacting Proteins by Mass Spectrometry

To identify ERG-interacting proteins that may serve as rational therapeutic targets and explore the mechanism by which ETS gene fusions mediate their effects, we performed mass spectrometric (MS) analysis of proteins that interact with the most prevalent ETS gene fusion product, ERG (encoded from *TMPRSS2* exon 1 fused to *ERG* exon 2; Tomlins et al. [2005]). VCaP prostate cancer cells (which harbor a *TMPRSS2:ERG* rearrangement) or human embryonic kidney (HEK) 293 cells were infected with either adenoviral V5 or FLAG epitope-tagged ERG expression vectors, respectively. Immunoprecipitation (IP) was completed in eight biological replicates to isolate protein-protein interactions as described by schematic (see Figure S1A available online). As expected, the interaction bait, ERG, was the top-scoring protein identified in the pull-down with 64.4% coverage with 17 tryptic peptides scanned over 500 times (Figure 1A; Table S1). Interestingly, three of the next four interacting proteins of high confidence and high sequence coverage identified were components of the DNA-dependent protein kinase complex and included the large catalytic subunit of a phosphatidylinositol 3/4 (PI3/4)-kinase called DNA-dependent protein kinase (DNA-PKcs) (10% coverage) and its known interacting subunits Ku70 (26% coverage) and Ku80 (34% coverage) (Figure 1A; Table S1). Interactions were confirmed with an independent antibody (Figure S1B), and IPs performed from VCaP cells demonstrated an endogenous association that occurs in the absence of ectopic overexpression (Figure 1B; Figure S1C).

To identify additional proteins participating in the ERG:DNAPK complex, we assessed our list of ERG interactors for other proteins known to interact with DNA-PKcs, Ku70, or Ku80 and identified two peptides for poly (ADP-ribose) polymerase 1 (PARP1): VVSEDFLQDVSASTK and QQVPSGESAILDR. Importantly, we demonstrated that PARP1 endogenously associated with ERG in VCaP cells (Figure 1B). We then performed reverse IPs using antibodies against DNA-PKcs, PARP1, and Ku80 and showed that each antibody was able to detect ERG-V5 protein (Figure S1D). To detect the PARP1:ERG interaction with the endogenous *TMPRSS2:ERG* gene fusion product, we used agarose-coupled PARP1 antibody to perform the IP-western, which confirmed that PARP1 interacts with the gene fusion product in an endogenous setting (Figure S1E).

Because DNA-PKcs only binds with Ku70 and Ku80 in the presence of DNA (Spagnolo et al., 2006), we tested the dependence of the ERG:PARP1 and ERG:DNA-PKcs interactions on intact DNA by performing the IP in the presence of 100 μM ethidium bromide. This treatment disrupted the interaction between ERG, Ku70, and Ku80, but not the interaction between ERG and either PARP1 or DNA-PKcs, demonstrating that the ERG:PARP1 and ERG:DNA-PKcs interactions are DNA independent (Figure 1B). As a control, we tested whether ERG would bind another PI3/4 kinase family member, ATR, or another protein known to interact with the DNAPK complex, XRCC4. Consistent with our IP-MS data, we were unable to detect an interaction between ATR or XRCC4 and ERG by IP-western blot analysis (Figure S1C and Figure 1B, respectively).

We next assessed whether the ERG:PARP1 and ERG:DNA-PKcs interactions occur in human prostate cancer tissues. ERG-IP showed enrichment for DNA-PKcs, Ku70, Ku80, and PARP1 in ERG gene fusion-positive, but not in ETS gene fusion-negative, prostate cancer tissues (Figure 1C; Figure S1F). Interestingly, the lack of detectable ERG:PARP1 interaction in tissue without ETS gene rearrangement is likely due to the near-absent ERG expression in rearrangement-negative prostate cancer (Park et al., 2010), as when overexpressed, wild-type (WT) ERG interacts with PARP1 in cell lines that do not

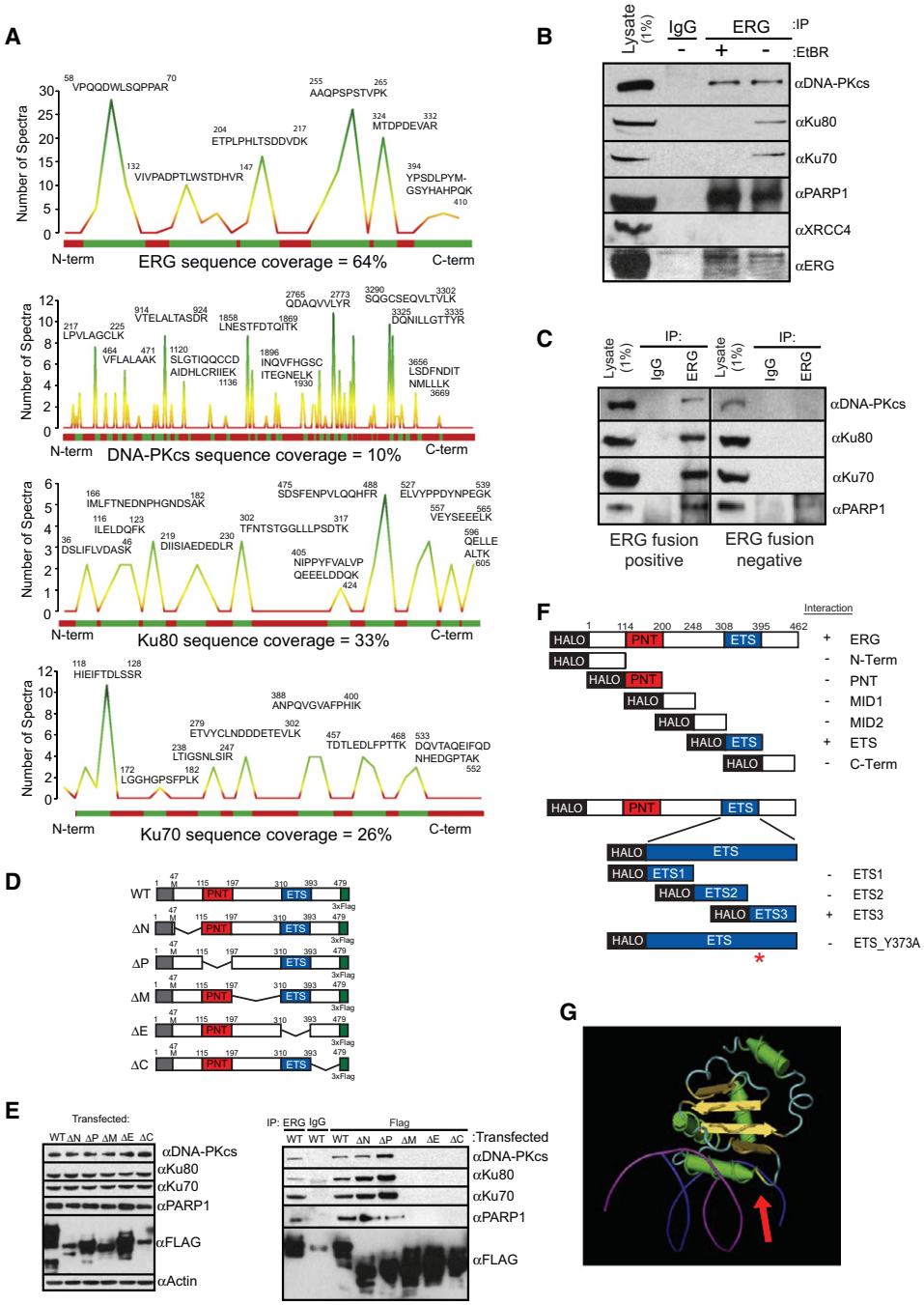

Figure 1. The *TMPRSS-ERG* Gene Fusion Product Interacts with PARP1 and the DNA-PK Complex

(A) MS analysis of proteins interacting with ERG. Histograms show peptide coverage of ERG, DNA-PKcs, Ku70, and Ku80.

(B) ERG, DNA-PKcs, PARP1, but not Ku70 or Ku80, interact independent of DNA. IP performed from VCaP cells that naturally harbor the ERG translocation.

(C) ERG, DNA-PKcs, PARP1, Ku70, and Ku80 associate in ERG gene fusion-positive human prostate cancer tissues. Representative ERG-positive and -negative prostate cancers shown of three pairs of tissues.

(D) Schematic of *TMPRSS2-ERG* gene fusion tiling deletion expression vectors.

(E) IP of DNA-PKcs, Ku70, Ku80, and PARP1 from HEK293 cells transfected with ERG expression vectors depicted in (D). Input western is shown on the left and IP-western shown on the right. All IPs were performed with FLAG antibody unless otherwise indicated.

(F) Schematic representation of halo-tagged ERG fragment vectors. The constructs were transcribed using wheat germ extracts, and halo-tagged protein was purified. Proteins were then incubated with purified DNA-PKcs and IP-westerns were performed. Fragments able to IP DNA-PKcs are indicated with a "+."

(G) Arrow indicates Y373, the amino acid required for the ERG:DNA-PKcs interaction. ETS1:DNA crystal from Garvie et al. (2001) used to demonstrate physical location of Tyrosine373 (from ERG) relative to the DNA-binding residues. One percent of the total cell lysate used for IP was added to the input lane. Representative experiments are shown.

See also Figure S1 and Table S1.

harbor the translocation (Figure S1G). Additional IP-westerns were performed to test the dependence of the ERG:PARP1 interaction on DNA in human prostate cancer tissues. Importantly, the interaction occurred in the absence of DNA in all three independent human tissues (Figure S1H).

Next, we sought to map the interactions and created a series of flag-tagged ERG expression vectors with tiling deletions, including: the N terminus (deletion of aa 47–115, predicted molecular weight 44.6 kDa); pointed domain (aa 115–197, 43.4 kDa); the middle amino acids (197–310, 41.6 kDa); the ETS domain (aa 310–393, 43.7 kDa); or the C terminus (aa 393–479, 43.6 kDa), and labeled the constructs ΔN, ΔP, ΔM, ΔE, and ΔC, respectively, as depicted in Figure 1D. IP following transient transfection demonstrated that the interactions between ERG, DNA-PKcs, Ku70, Ku80, and PARP1 occurred in the C-terminal half of the ERG protein (Figure 1E). To further map the ERG:PARP1 and ERG:DNA-PKcs interactions and to confirm that both PARP1 and DNA-PKcs interact with other ETS family member proteins, we performed IP-western blot analysis in HEK293 cells transfected with *ERG-FLAG*, *ETS1-FLAG*, *SPI1-FLAG*, or *ETV1-FLAG* expression vectors, which were selected for their sequence relationship to ERG (Figure S1I). In all four experiments, pull-downs confirmed the interactions (Figures S1G and S1J–S1L). We then created N-terminal halo-tagged expression vectors for in vitro purification of these ETS genes. Subsequent IP-westerns demonstrated that all four of these proteins bind directly to DNA-PKcs (Figure S1M). Given the sequence alignment of these four ETS proteins and the large tiling deletion data, our data suggested that the interactions occur through the ETS DNA-binding domain.

To definitively map the ERG:DNA-PKcs and ERG:PARP1 interactions, we utilized HALO-tagged WT ERG and six individual HALO-tagged fragments spanning the entire ERG protein (Yu et al., 2010). As expected, IP-western blot demonstrated that the ERG:DNA-PKcs interaction occurred through the ETS DNA-binding domain. To further map the interaction between ERG and DNA-PKcs, we utilized a series of three HALO-tagged fragments that tiled the ETS domain, which localized the interaction to the final 28 amino acids of the ETS domain (Figure 1F; Figure S1N). Importantly, although, to our knowledge, the crystal structure of the ETS domain from ERG has not yet been reported, the crystal structure of another ETS factor that we demonstrated interacts with DNA-PKcs, ETS1, has been published (Garvie et al., 2001). Based on homology with other interacting ETS proteins and structural information, we predicted that the interaction was dependent on the amino acids, YYDKN. By site-directed mutagenesis of each residue to alanine, we demonstrated that the Y373A mutant was unable to precipitate DNA-PKcs, suggesting that this interaction is mediated by Tyrosine 373 (Figure 1F; Figure S1P). Analysis of the ETS1 structure shows that Y373 is adjacent to the arginine residues that fit into the DNA groove and that Y373 is accessible to potential interacting proteins (Figure 1G).

After demonstrating that ERG interacts with DNA-PKcs directly through amino acid Y373, we sought to map the ERG:PARP1 interaction. However, purified ERG was only able to interact with purified PARP1 in the absence of ethidium bromide (Figure S1O). Because the interaction occurred in cells independent of ethidium bromide, this suggests that the ERG:PARP1 interaction is mediated by other proteins. This is consistent with the results from our IP-MS experiment in which few PARP1 peptides were identified, suggesting that the ERG:PARP1 interaction is mediated by an intermediate protein such as DNA-PKcs.

PARP1 and DNA-PKcs Are Required for ERG-Mediated Transcription

Given that the interaction of DNA-PKcs and PARP1 with ERG occurs through the ETS domain, we hypothesized that both PARP1 and DNA-PKcs function as coregulators of ERG transcriptional activity. Thus, we performed chromatin immunoprecipitation (ChIP) assays in VCaP cells and assessed enrichment of known ERG targets, including the *PLA1A* promoter and the *FKBP5*, *PSA*, and *TMPRSS2* enhancers. These experiments demonstrated that ERG, DNA-PKcs, activated DNA-PKcs (assessed by T2609 phosphorylation), Ku70, Ku80, and PARP1 bind to these sites, but not to the negative control gene *KIAA0066* (Tomlins et al., 2008) (Figure S2A). Interestingly, this enrichment was disrupted by ERG siRNA (Figure 2A; Figure S2B), supporting a model in which ERG recruits PARP1 and DNA-PKcs to specific genomic loci during transcription (Figure 2B). Consistent with this hypothesis, serial ChIP reactions (ERG, then PARP1 or DNA-PKcs) demonstrated that an ERG:PARP1 complex and an ERG:DNA-PKcs complex are both present at ERG-regulated loci (Figure S2C). Although it was not possible to perform re-ChIP experiments with the PARP1 and DNA-PKcs antibodies, IP-western blot analysis confirmed that PARP1 and DNA-PKcs interact in a DNA-independent manner in VCaP cells (Figure S2D). Likewise, this experiment suggests that DNA-PKcs binding to ERG does not disrupt the ERG:DNA interaction.

To test whether DNA-PKcs and PARP1 are required for ERG-mediated transcriptional activation, we constructed a *PLA1A* promoter reporter. Transfection of the reporter into RWPE cells treated with either LACZ or ERG adenovirus and siRNA (Figure S2E) indicated that both DNA-PKcs ($p = 1.99 \times 10^{-3}$) and PARP1 ($p = 2.37 \times 10^{-3}$) are required for ERG-induced activation of *PLA1A* (Figure S2E) in RWPE cells. In contrast, inhibition of the related PI3/4-like kinase, ATM, had no significant effect on ERG activity.

Although ATM and ATR repair DNA strand breaks through different pathways, DNA-PKcs is specifically required for nonhomologous end-joining (NHEJ) (Weterings and Chen, 2007). In this process, DNA-PKcs, Ku70, and Ku80 form a complex on the broken DNA end that facilitates DNA end processing and rejoining in a multistep procedure that requires the XRCC4/DNA Ligase IV complex. In fact, XRCC4 and DNA Ligase IV are both independently required for execution of NHEJ in mammalian cells because targeted inactivation of either gene leads to NHEJ defects in mouse cells (Barnes et al., 1998; Frank et al., 1998). Subsequently, we used siRNA to knockdown XRCC4 (Figure S2E) to evaluate the necessity of effective execution of NHEJ for ERG-induced transcriptional activation of the *PLA1A* promoter. Because knockdown of XRCC4 had no effect on ERG activity, the experiment further suggests a NHEJ-independent role for DNA-PKcs in ERG-mediated transcription (Figure S2E).

Given the importance of PARP1 and DNA-PKcs for ERG-mediated transcription, we sought to explore the global effects of inhibiting PARP1 and DNA-PKcs on the ERG transcriptome.

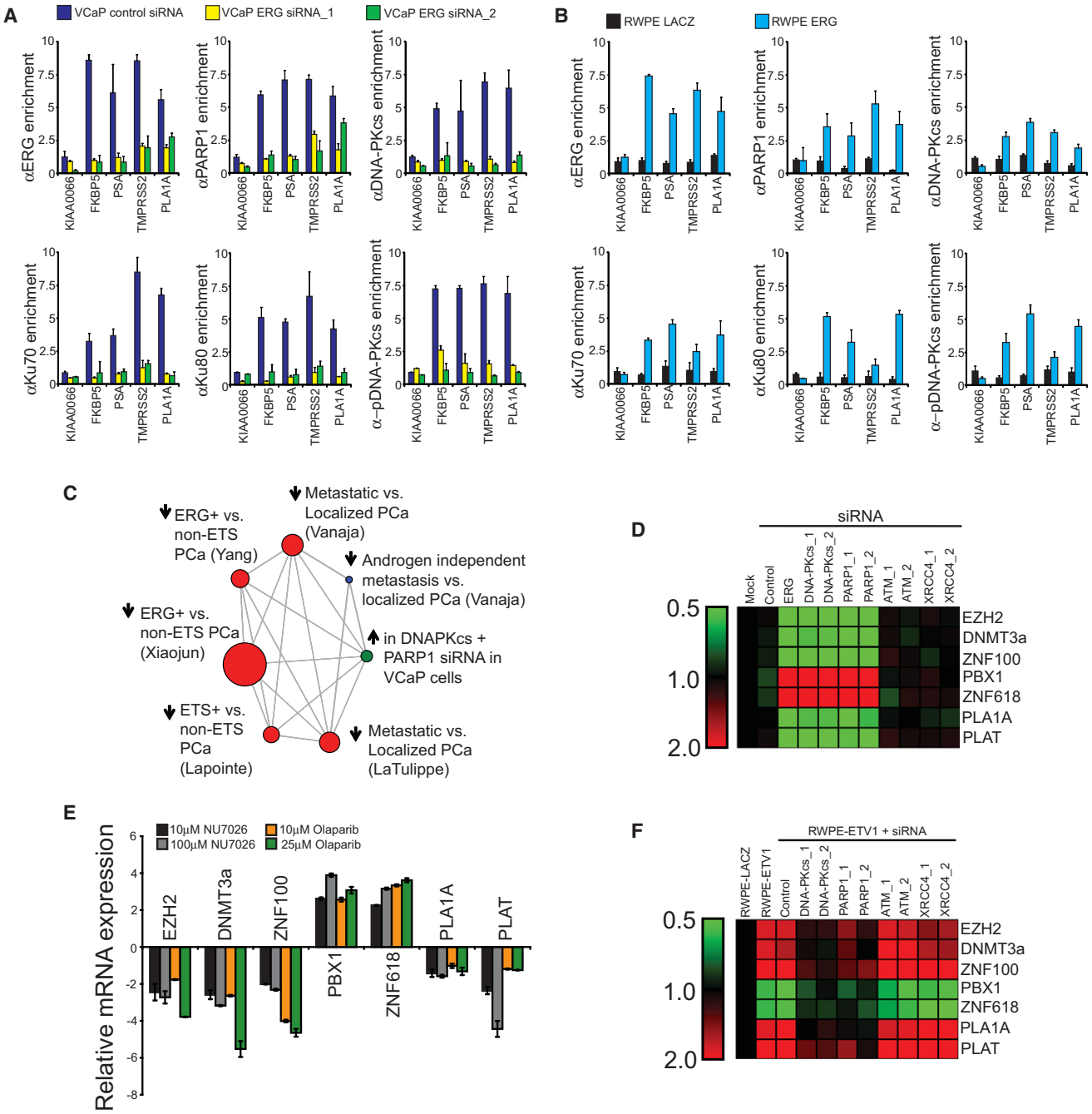

Figure 2. PARP1 and DNA-PKcs Are Required for ERG-Regulated Transcription

(A) ChIP of PARP1 and the DNAPK complex shows an association with ERG-regulated targets, including the *PLA1A* promoter as well as *FKBP5*, *PSA*, and *TMPRSS2* enhancers, but not the negative control gene *KIAA0066*. ChIPs were performed in VCaP cells treated with control or one of two independent ERG siRNAs for 48 hr prior to crosslinking.

(B) ChIP performed as in (A) but with stable RWPE-ERG or -LACZ cells.

(C) Data from gene expression arrays were analyzed by molecular concept mapping. The gene set analyzed is the set of genes that was greater than 2-fold differential in all three siRNA treatments relative to control. This gene set was used to determine the correlation of genes regulated by ERG, DNA-PKcs, and PARP1 in VCaP cells with published microarray data. Node size is proportional to the number of genes in the set, and edges represent statistically significant associations ($p < 0.01$). Arrow directionality represents gene sets either being induced or repressed.

(D) VCaP cells were treated with siRNA as indicated 48 hr prior to RNA isolation. qPCR was then run to confirm gene expression changes identified in the microarray experiment. Data are shown as a heat map with siRNA treatments along the x axis and genes expression analyzed by qPCR along the y axis. Shades of green represent downregulation of gene expression, whereas shades of red represent upregulation.

(E) VCaP cells were treated with either NU7026 or Olaparib for 48 hr as indicated, and qPCR analysis of ERG target genes identified from gene expression microarray experiment was performed.

To do this, we used Agilent Whole Genome Oligo Expression Arrays to profile RNA from VCaP cells treated with either DNA-PKcs or PARP1 siRNA (knockdown confirmed in Figure S2I). Our analysis revealed 50 and 252 unique features that were greater than 2-fold down- and upregulated, respectively, in both the PARP1 and DNA-PKcs siRNA-treated samples (Table S2). Venn diagram analysis was used to show the overlap of differential gene sets to genes regulated by ERG in VCaP cells (Tomlins et al., 2008) ($p < 0.0001$ for all interactions unless indicated, hypergeometric test) (Figures S2F and S2G). To then understand how this gene signature is related to existing signatures, we uploaded our expression signature into Oncomine Concepts Map (OCM) (Rhodes et al., 2007; Tomlins et al., 2007b) to identify human tissue gene signatures that are enriched for genes upregulated by DNA-PKcs and PARP1 siRNA in VCaP cells (genes repressed by PARP1 and DNA-PKcs). This provided unbiased validation that the tissue-based gene signatures most highly enriched with our gene set were the genes repressed in ETS-positive as compared to ETS-negative prostate cancer: Tomlins et al. (2007b) (OR = 3.08, $p = 1.40 \times 10^{-15}$) and (OR = 2.91, $p = 3.30 \times 10^{-10}$); and Lapointe et al. (2004) (OR = 3.33, $p = 2.30 \times 10^{-6}$) (Figure 2C). Interestingly, the gene signature also showed significant overlap with the set of genes repressed in metastatic as compared to localized prostate cancer, suggesting that repression of these genes is important for prostate cancer progression: OR = 2.99, $p = 1.5 \times 10^{-10}$ (Vanaja et al., 2003); OR = 3.31, $p = 1.50 \times 10^{-6}$ (LaTulippe et al., 2002) (Figure 2C). Treatment of VCaP cells with siRNA confirmed gene expression changes as predicted by the gene expression arrays (Figure 2D), as did treatment with either the small molecule DNA-PKcs kinase inhibitor, NU7026, or the small molecule PARP1 inhibitor, Olaparib (Figure 2E). Analysis of siRNA-treated RWPE-ETV1 cells (Figure S2J) confirmed that DNA-PKcs and PARP1 regulated ETV1 transcriptional activity as well (Figure 2F). Taken together, these data suggest that PARP1 and DNA-PKcs play a role in modulating transcriptional activity of a number of ETS target genes, some of which are differentially expressed between localized and metastatic disease.

PARP1 and DNA-PKcs Are Required for ERG-Mediated Cell Invasion, Intravasation, and Metastasis

Inhibition of PARP1 and DNA-PKcs altered ERG transcriptional activity of several progression-associated genes such as *EZH2*. Here, we tested the role of these enzymes in ERG-induced cell invasion. Both DNA-PKcs siRNA (Figures S2H and S2I) and NU7026 attenuated invasion in RWPE cells transduced with ERG (Figure 3A) and VCaP cells (Figure 3B) ($p < 0.01$ for DNA-PKcs siRNA or NU7026 >10 μM). Likewise, we found that treatment with either PARP1 siRNA (Figures S2H and S2I) or Olaparib led to a significant reduction in ERG-driven RWPE and VCaP cells invasion (Figures 3A and 3B) ($p < 0.05$ for all PARP1 siRNA or Olaparib treatments). As with our analysis of ERG-mediated transcription, knockdown of either ATM or XRCC4 did not have an effect on ERG-mediated invasion (Figures 3A and B). Treatment of stable RWPE cells stably overexpressing ETV1 (Tomlins et al., 2007a) with PARP1 or DNA-PKcs siRNA (Figure S2J) or small molecule inhibitors also led to a significant reduction in invasion ($p < 0.01$ for all PARP1 or DNA-PKcs treatments) (Figure 3C). However, importantly, invasion of two negative control models, the ETS rearrangement-negative cell line PC3 and RWPE cells overexpressing an alternative prostate cancer gene fusion, *SLC45A3-BRAF*, was not affected by inhibiting either enzyme (Figure 3D; Figure S2K). To determine if the observed loss of cell invasion was due to cytotoxicity, we performed chemosensitivity assays with both Olaparib and NU7026. Neither Olaparib nor NU7026 had an effect on the in vitro cell proliferation rate of any of the cell lines tested, suggesting that the reduction in cell invasion is not due to changes in cell proliferation (Figure S3A). In fact the EC_{50} for both drugs was well beyond the dose shown to block transcription and invasion (Figures S3B and S3C).

We next sought to define the role of PARP1 in ERG-mediated invasion and intravasation in vivo. To do this, we implanted cells onto the upper chorioallantoic membrane (CAM) of a fertilized chicken embryo and analyzed the relative number of cells that invade and intravasate into the vasculature of the lower CAM 3 days after implantation (Kim et al., 1998). In this assay, Olaparib blocked both ERG-mediated invasion and intravasation ($p < 0.1$) (Figures 4A and 4B). Because increased expression of *EZH2* alone is sufficient to drive metastasis in several different cell systems, we monitored *EZH2* mRNA expression and found that *EZH2* expression was downregulated following either PARP1 or DNA-PKcs inhibition (Figure S4A), suggesting that mechanistically, PARP inhibition disrupts ERG-mediated invasion and intravasation by inhibiting ERG-mediated transcriptional activation of invasion-associated genes such as *EZH2*. However, more importantly, our models suggested that therapeutic disruption of either ERG-interacting enzymes (PARP1 or DNA-PKcs) inhibits the metastatic spread of prostate cancers harboring ETS gene fusions.

To test this postulate, we analyzed the metastatic potential of an ETS-positive (LNCaP) and an ETS-negative (PC3) cell line. As shown in Figure 4C, Olaparib treatment blocked the formation of liver metastases from LNCaP ($p = 0.01$), but not PC3 cells. Importantly, we also noticed that over the extended treatment period, the ETS-positive tumors were significantly smaller than the ETS-negative tumors (Figure 4D), with $p < 0.05$ for VCaP and $p < 0.01$ for LNCaP. This suggests that PARP1 could play a role in the long-term maintenance of ETS-positive cancer cell survival. Because it appeared that the long-term survival of ETS-overexpressing tumors can be diminished by treatment with Olaparib, we sought to compare the magnitude of effect to that of a clinically validated model. Therefore, we xenografted HCC1937 (BRCA1 mutant) and MDA-MB-231 (BRCA1/2 WT) cell lines, and following Olaparib treatment a significant effect was observed on the BRCA1 mutant HCC1937 tumors, whereas no measurable effect was observed in MDA-MB-231 tumors. Surprisingly, the magnitude of effect observed in the HCC1937 cells was equivalent to the magnitude of effect observed in the two ETS-positive cell line xenografts (Figure 4D).

(F) As in (D) except stable RWPE-ETV1 cells were used. All qPCR experiments were run three times in quadruplicate. All bar graphs are shown with ±SEM unless otherwise indicated.
See also Figure S2 and Table S2.

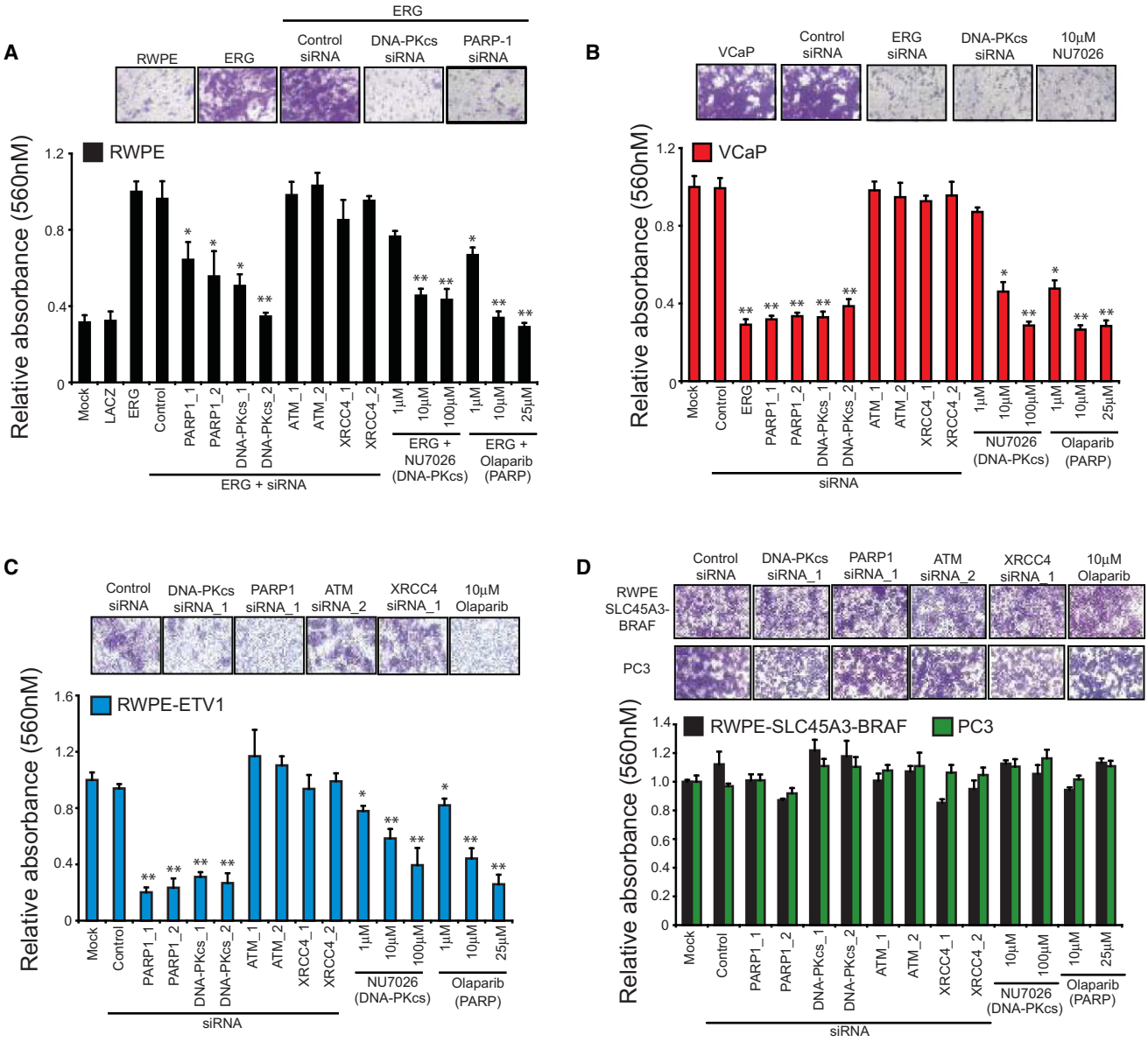

Figure 3. ERG-Mediated Invasion Requires Engagement of PARP1 and DNA-PKcs

(A) RWPE cells were infected with ERG adenovirus and treated with indicated siRNAs or different doses of the DNA-PKcs inhibitor, NU7026, or the small molecule PARP1 inhibitor, Olaparib, for 48 hr prior to plating cells in Matrigel-coated Boyden chambers. After another 48 hr, cell invasion was quantified.

(B) As in (A) except VCaP cells.

(C) As in (A) except stable RWPE cells transduced with ETV1 lentivirus.

(D) As in (A) except PC3 or RWPE-SLC45A3-BRAF cells. Representative of three independent experiments. Representative photomicrographs of invaded cells are shown (lower Boyden chamber stained with crystal violet). For all experiments mean ± SEM is shown (*$p < 0.05$, **$p < 0.01$).

See also Figure S3.

ETS Gene Fusion Prostate Tumors Are Preferentially Susceptible to PARP1 Inhibition In Vivo

Based upon our in vivo data from the chicken CAM assay, we hypothesized that inhibition of PARP1 would inhibit ETS-positive prostate cancer growth in mouse xenograft models. Several PARP inhibitors have entered phase I and phase II clinical trials (Audeh et al., 2010; O'Shaughnessy et al., 2011; Tutt et al., 2010). One of these, Olaparib, was shown to be well tolerated with a minimal side effect profile in patients with cancer (Fong et al., 2009). Thus, to first test our hypothesis, we implanted VCaP (ERG positive) or PC3-LACZ (ETS negative) cells and studied the impact of Olaparib (100 mg/kg/day, IP) on xenograft growth. Importantly, we observed a significant reduction of tumor growth in the ETS-rearranged cell line relative to that of the vehicle control, but no change was observed in the ETS-negative control cell line ($p = 0.002$ for VCaP cells), suggesting preferential sensitivity of ETS-positive tumors (Figures S5A and S5B).

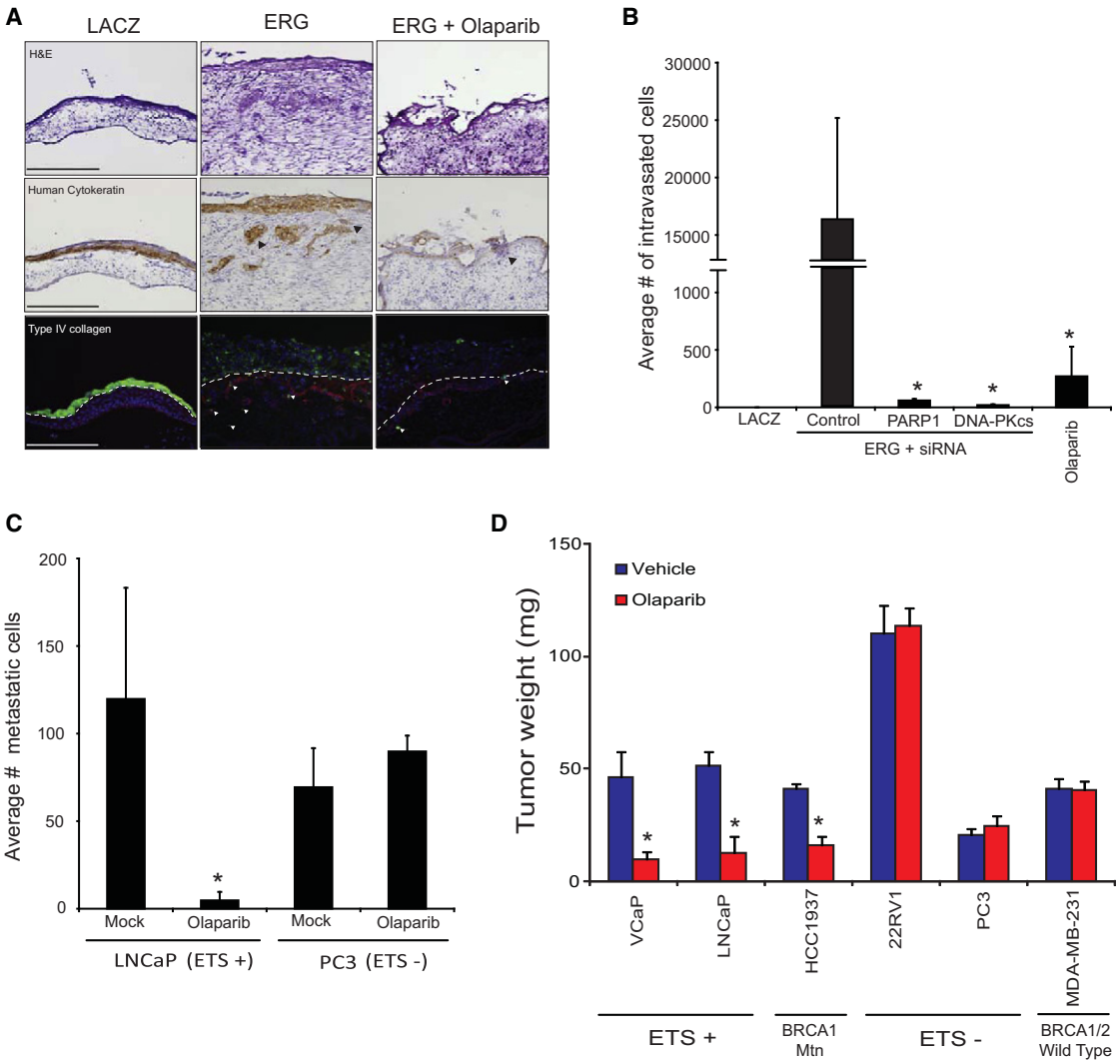

Figure 4. ERG-Mediated Invasion, Intravasation, and Metastasis Require PARP1 Activity In Vivo

(A) CAM invasion assay performed using stable RWPE-LACZ, or RWPE-ERG cells labeled with microspheres (green fluorescence emission) and treated with or without a single dose of Olaparib (40 mg/kg) as indicated. Seventy-two hours after implantation, the upper CAM was harvested. Frozen sections were created and stained for hematoxylin and eosin (top row), human-specific cytokeratin (immunohistochemistry, middle row), or chicken-specific type IV collagen (red immunofluorescence, bottom row). Arrowheads indicate cells invaded through the upper CAM. Representative images are shown. Scale bars, 200 μM.

(B) CAM intravasation assay performed using stable RWPE-ERG cells pretreated with siRNA as indicated. Alternatively, RWPE-ERG cells were implanted and treated with a single dose of Olaparib immediately after implantation (40 mg/kg). Seventy-two hours after implantation, the lower CAM was harvested. Total DNA was isolated from the lower CAM, and qPCR was performed using human-specific *ALU* PCR primers. Total cell number was determined by comparing to a standard curve created using varying amounts of RWPE cells as input.

(C) Liver metastasis in chicken embryos was assessed 8 days following implantation of either LNCaP (ETV1 rearrangement) or PC3 (no ETS rearrangement) cells onto the upper CAM. Animals were injected every other day with Olaparib (40 mg/kg) prior to harvesting chicken livers. Total cell number was then quantified by qPCR as in (B).

(D) ETS-positive (VCaP and LNCaP) and ETS-negative (PC3 and 22RV1) prostate cancer cells as well as BRCA1 mutant (HCC1937) and BRCA1/2 WT (MDA-MB-231) breast cancer cells were implanted onto the upper CAM. These cell line xenografts were then treated with 40 mg/kg Olaparib every other day for 8 days. Tumors (noninvaded cells remaining on the upper CAM) were collected and weighed. Average tumor weight is shown. For all experiments mean ± SEM is shown (*$p < 0.05$).

See also Figure S4.

We then extended our experiment to analyze the effects of Olaparib on a panel of ETS-positive and ETS-negative prostate cancer cell lines, including an isogenic model. Because this experiment intended to test the specificity of Olaparib-induced growth inhibition for ERG-overexpressing prostate xenografts, we chose to use a dose similar to that used in previously published xenograft experiments (Rottenberg et al., 2008). Consistent with our hypothesis, this dose of Olaparib had a significant effect on VCaP cells ($p = 0.001$) but did not inhibit the growth of two additional ETS-negative cell line xenografts (22RV1 or

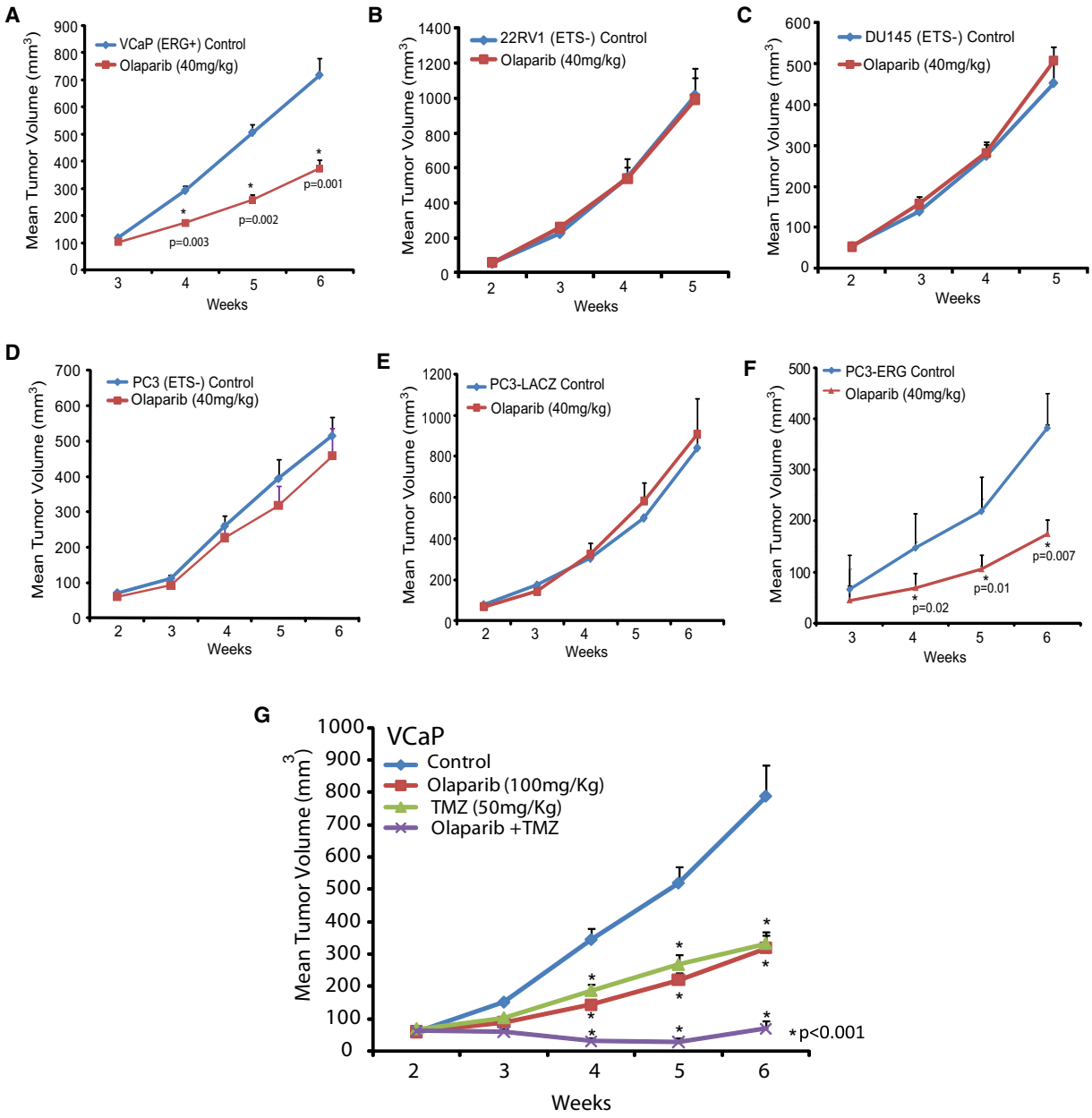

Figure 5. Inhibition of PARP1 Alters ETS-Positive, but Not ETS-Negative, Cell Line Xenograft Growth

(A–F) Specificity screen for ETS-positive and ETS-negative tumor cell line xenografts. Cell lines were injected subcutaneously and grown until tumors were palpable. Xenografted mice then received i.p. injections of Olaparib 40 mg/kg as indicated 5 days/week. Caliper measurements were taken weekly. ETS-positive cell line xenografts were (A) VCaP (ERG rearrangement) and (F) PC3-ERG cells, and the ETS-negative xenografts were (B) 22RV1, (C) DU145, and (D and E) PC3-Control/-LACZ, respectively.

(G) Mice xenografted with VCaP cells were treated as in (A) except with 100 mg/kg Olaparib and/or 50 mg/kg TMZ as indicated. Olaparib was administered i.p. 5 days/week. TMZ was administered in two 5 day cycles with the first occurring during week 3 and the second occurring during week 5. For all experiments mean ± SEM is shown (*$p < 0.01$ unless indicated).

See also Figure S5.

DU145) (Figures 5A–5C). This experiment also demonstrated that the VCaP tumor growth response was dose dependent. To then create an isogenic model, we overexpressed the primary *TMPRSS2:ERG* gene fusion product in the PC3 prostate cancer cell line (PC3-ERG). Western blotting confirmed protein overexpression, and IP-western confirmed the ERG:DNA-PKcs and ERG:PARP1 interactions (Figures S5C and S5D). ChIP assays demonstrated that ERG binds to known target genes in PC3 cells (Figure S5E), and qPCR demonstrated ERG transcriptional activity (Figure S5F). Surprisingly, ERG overexpression led to a slightly reduced growth rate of PC3 cells relative to LACZ-overexpressing PC3 cells (Figure S5G). Consistent with the model

that ETS-positive, but not ETS-negative, prostate tumors are susceptible to PARP inhibition, overexpression of ERG was sufficient to significantly sensitize PC3 cells to PARP inhibition ($p = 0.007$), suggesting that ERG overexpression provides a selective mechanism for Olaparib-mediated growth inhibition (Figures 5D–5F). Western blot and qPCR analysis of flash-frozen, staged PC3-ERG tumors treated with or without drug for 4 hr confirmed inhibition of PARP1 activity and loss of ERG-target gene expression after treatment with Olaparib (Figures S5H–S5K). In line with the clinical observation that Olaparib is well tolerated at doses capable of inhibiting PARP activity (Fong et al., 2009), Olaparib treatments in our xenograft models did not lead to a significant decrease in total body weight and did not lead to liver toxicity, as assessed by serum levels of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) (Figure S5L).

Given the specific effect of Olaparib on ERG-positive cell line xenograft growth (Figure S5M), we extended our analysis with the use of a model of primary human prostate tumors maintained in serial xenografts (Li et al., 2008). We identified one ERG-positive (MDA-PCa-133), one ETV1-positive (MDA-PCa-2b-T) (FISH confirmed; Tomlins et al., 2007a), and one ETS-negative (MDA-PCa-118b) model for this experiment by assessing relative levels of ETS gene expression by qPCR (Figure S5N). Functional ETS gene status was assessed by testing the relative expression of several ETS target genes, including *EZH2*, *DNMT3a*, *ZNF100*, *PBX1*, *ZNF618*, *PLA1A*, and *PLAT*, between the models (Figure S5O). As shown in Figures S5P and S5Q, low-dose Olaparib altered the growth of both the ERG and ETV1-overexpressing primary human prostate cancer xenografts (MDA-PCa-133: $p = 0.05$ [day 8] and $p < 0.01$ [days 12, 16, 20, 24, 28, and 32]; MDA-PCa-2b-T: $p < 0.01$ [days 8, 12, and 16]) but had no effect on the ETS-negative primary human xenograft model (Figure S5R). In all cases, Olaparib did not have an observable effect on total body weight (Figures S5S–S5AA).

Because we were able to validate the hypothesis that Olaparib specifically alters the growth of either ETS-overexpressing or BRCA1/2-deficient cell lines, we sought to extend the treatment regimen to identify combination therapies that enhance the magnitude of inhibition without causing significant toxicity. Recently, an alkylating agent called temozolomide (TMZ) has been shown to potentiate the effects of other PARP inhibitors in several cancer xenograft models (Donawho et al., 2007; Liu et al., 2008; Palma et al., 2009) as well as caused a complete or partial response in some patients enrolled in a phase II trial for metastatic breast cancer (S.J. Isakoff et al., 2010, J. Clin. Oncol., abstract). As expected, the combination treatment resulted in a significant growth reduction of VCaP tumors that was maintained over the entire 6 weeks ($p < 0.001$ for all combination treatments) (Figure 5G). Even with the combination therapy, at this dose range, no overt toxicity such as excessive weight loss was observed (Figure S5AB). This suggested that the addition of PARP inhibitor therapy to existing chemotherapeutic regimens will help enhance the overall effect for ETS-positive tumors.

ETS Transcription Factors Drive DNA Double-Strand Break Formation

To explore potential mechanisms of ETS-specific therapeutic response to these inhibitors of DNA repair, we assessed total levels of DNA double-strand breaks in vitro. We hypothesized that constitutive overexpression of ERG may lead to an increased susceptibility to DNA damage. Thus, we first assessed the total levels of a histone mark of DNA double-strand breaks called γ-H2A.X in Olaparib-treated versus untreated VCaP cells. Surprisingly, the untreated cells had a high level of γ-H2A.X foci (Figure 6A), leading us to test the hypothesis that overexpression of ETS genes induces DNA double-strand breaks. Overexpression of ETS genes in primary PrECs induced >5 γ-H2A.X foci in greater than 75% of the analyzed cells, whereas the control genes *LACZ* and *EZH2* had no effect (Figures 6A and 6B). Quantitative PCR confirmed overexpression (Figures S6A–S6C). Likewise, other ETS genes were also capable of inducing γ-H2A.X foci in several different prostate cell lines (Figures S6D and S6E). To then confirm that ERG induces γ-H2A.X foci in an endogenous setting, we depleted ERG from VCaP cells by RNA interference (Figure S6I) and found a significant decrease in the average number of γ-H2A.X foci ($p = 7.16 \times 10^{-3}$ and $p = 1.36 \times 10^{-3}$) for two independent siRNAs, respectively (Figures 6A and 6B). Although γ-H2A.X foci represent an early mark of DNA-damage recognition, 53BP1 is present only in the later stages of repair (Bennett and Harper, 2008). As such, in the presence of a DNA-damage response, we expected to observe an increase in 53BP1 foci formation. Indeed, the ETS genes also induced 53BP1 foci formation (Figures 6A and 6B; Figure S6D).

After demonstrating that ETS gene overexpression drives the accumulation of markers of DNA double-strand breaks, we sought to confirm the presence of DNA double-strand breaks by directly analyzing cellular DNA for fragmentation using the COMET assay. As with the γ-H2A.X and 53BP1 foci formation assays, in PC3 cells, ERG or ETV1 overexpression was sufficient to induce significantly longer and brighter tails than those observed in controls ($p < 0.01$ for both ETS genes), and treatment with either ERG siRNA led to a reduction in relative level of DNA double-strand breaks ($p < 0.01$) (Figures 6C and 6D).

Olaparib Potentiates ETS-Induced DNA Damage

After finding that aberrantly expressed ETS transcription factors drive the accumulation of DNA double-strand breaks, we hypothesized that by having a baseline level of DNA damage, ETS-positive cancers may be specifically susceptible to accumulating DNA damage following inhibition of the interacting DNA-repair enzyme PARP1. To test this hypothesis, we analyzed VCaP cells treated with Olaparib for 48 hr. Olaparib-treated VCaP cells had a very high level of γ-H2A.X foci (Figure S6F). Importantly, by depleting endogenous ERG using either of two independent siRNAs (confirmed in Figure S6I), we were able to reverse the gross increase in γ-H2A.X. Similar increases in foci were observed in PC3-ERG cells or PC3 cells with BRCA2 knockdown, but not in the control cells (Figure S6F). Knockdown efficiency was confirmed by qPCR (Figure S6G). Quantification of the relative levels of DNA double-strand breaks demonstrated that, whereas there was an increase in the tail moment of all Olaparib-treated cell lines, Olaparib caused a significantly greater increase in the tail moments of ERG-positive cells than controls ($p < 0.001$, two-way ANOVA) (Figures 6C and 6D). In conjunction with this observation, both ERG siRNAs led to a significant reduction in DNA damage following Olaparib and blocked the synergistic increase of DNA damage observed with Olaparib

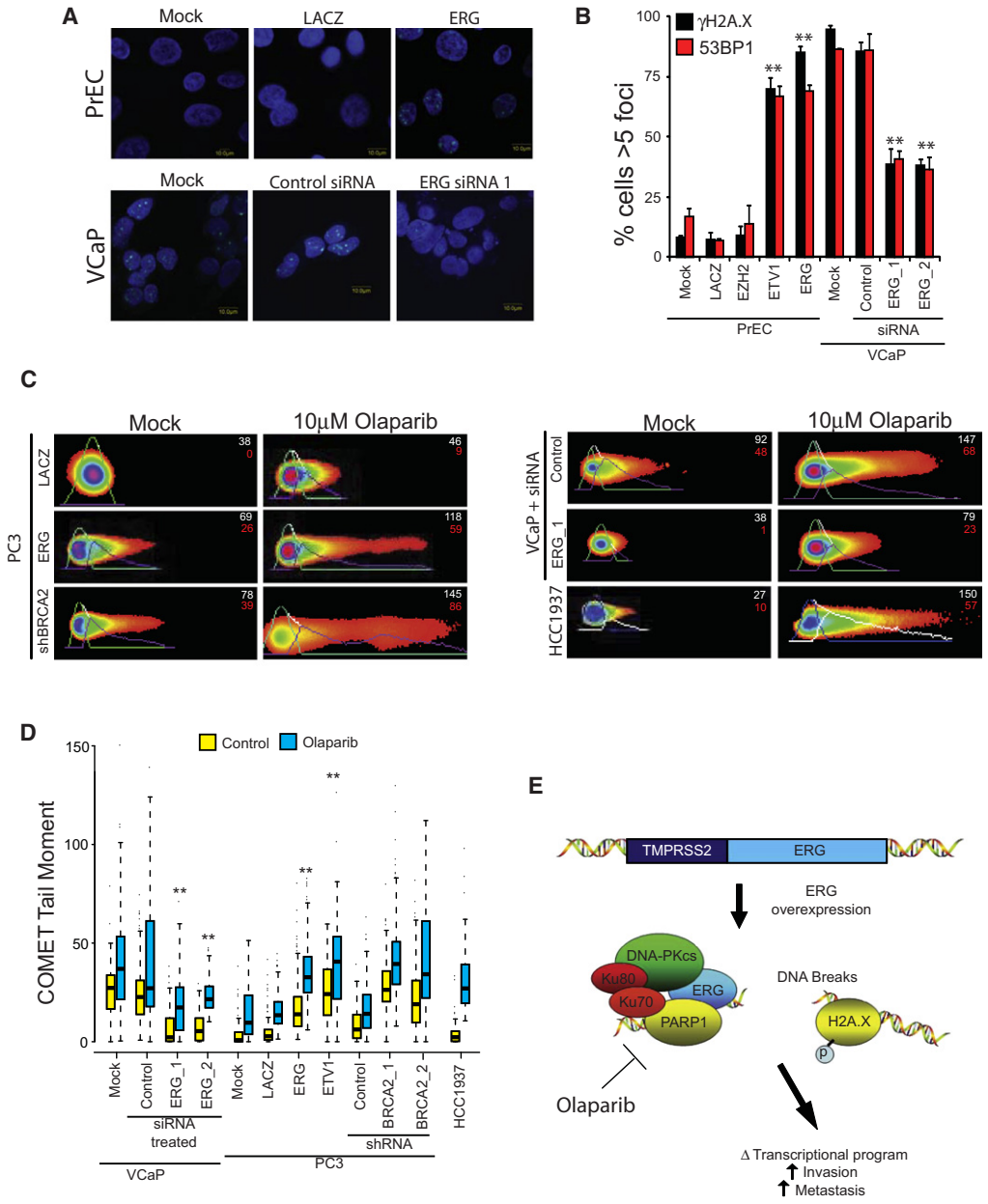

Figure 6. ETS Transcription Factors Induce DNA Damage that Is Potentiated by PARP Inhibition

(A) γ-H2A.X immunofluorescence staining shows that ERG induces the formation of γ-H2A.X foci. Top row shows benign PrECs were infected with lentiviruses expressing LACZ or ERG. Bottom row illustrates VCaP cells treated with control siRNA or ERG siRNA.

(B) Quantification of γ-H2A.X and 53BP1 immunofluorescence staining in PrEC or VCaP cells. For all experiments mean ± SEM is shown (**$p < 0.01$).

(C) ETS overexpression or BRCA2 knockdown (with shRNA) induces DNA damage as assessed by neutral COMET assay in VCaP cells. Cells were treated with or without 10 μM Olaparib for 48 hr. Cells with DNA damage have an extended "tail moment" of fragmented DNA shown in red. Relative tail length is shown in white. Representative images showing quantification of head and tail height, length, and fluorescence intensity are shown (as indicated).

(D) Quantification of average COMET tail moments following treatment as noted in the box plot. Statistical tests were performed using the two-way ANOVA test (described in Supplemental Experimental Procedures) to determine if the increase in DNA damage in Olaparib-treated ETS-overexpressing cells (PC3-ERG, PC3-ETV1, and VCaP) was statistically greater than the increase observed in Olaparib-treated control cells with low ETS expression (PrEC-LACZ, PC3-LACZ, or VCaP treated with ERG siRNA) as indicated in the text. Similar statistical tests were used to compare the increase in BRCA2 shRNA-expressing cells to PC3 cells transduced with control shRNA (**$p < 0.01$).

(E) Proposed model to therapeutically target ETS gene fusions via their interacting enzyme, PARP1. All bar graphs are shown with ±SEM unless otherwise indicated.

See also Figure S6.

($p = 0.001$ for ERG siRNA_1 and $p < 0.001$ for ERG siRNA_2, two-way ANOVA). Consistent with this observation, the BRCA1 mutant HCC1937 breast cells and PC3 cells with BRCA2 shRNA underwent a significant increase in the total levels of DNA damage when treated with Olaparib. Taken together, these data demonstrate that similar to BRCA1/2-deficent tumors, ETS-positive, but not ETS-negative, prostate cancer models are susceptible to PARP inhibition through the increased incidence of DNA double-strand breaks (Figure 6E).

To discriminate between mechanisms of ERG-potentiated DNA damage, we performed the COMET assays after 0.5, 1, 24, and 48 hr exposure to Olaparib. Surprisingly, the potentiated DNA damage was observed in PC3-ERG cells relative to PC3-LACZ cells as early as 30 min after treatment (Figure S6H) ($p = 0.002$ at 30 min, two-way ANOVA). This suggested that the mechanism of ERG-induced potentiation is independent of the genes regulated by PARP1-mediated transcriptional activation. Focused expression analysis of genes involved in DNA-damage repair pathways demonstrated no significant change in any of the repair genes analyzed, suggesting that the DNA-damage phenomenon is independent of changes to ERG-regulated gene expression (Figure S6I). To analyze the role of repair pathways directly, we tested the postulate that downregulation of a protein critical for the execution of NHEJ pathway such as XRCC4 would lead to a synergistic induction of DNA damage in a homologous recombination (HR)-deficient cell. Treatment of HR-deficient HCC1937 cells with siRNA confirmed a greater increase in DNA damage following XRCC4 knockdown (NHEJ) than by XRCC3 knockdown (HR) ($p < 0.05$, one-way ANOVA). In contrast, the synergistic induction of DNA damage following XRCC4 or XRCC3 knockdown was not observed in PC3-ERG cells as compared to PC3-LACZ cells (Figures S6J and S6K). This suggested that ERG overexpression does not completely block either of these double-strand break repair pathways. This was further confirmed by HR-efficiency assays that demonstrated that HR is not significantly altered by ERG overexpression (Figure S6L).

DISCUSSION

In this study we discovered that the ETS gene fusion product, ERG, physically interacts with the enzymes PARP1 and DNA-PKcs. Both PARP1 and DNA-PKcs are required for ERG-mediated transcription and cell invasion, suggesting that both of these cofactors are necessary for ERG-mediated prostate cancer progression. Moreover, therapeutic inhibition of PARP1 preferentially sensitized ETS-overexpressing prostate cancer xenografts compared to ETS-negative xenografts. Thus, similar to the successful paradigm of targeting the BCR-ABL gene fusion in CML with the small molecule kinase inhibitor imatinib mesylate (Druker et al., 2001), one could envision targeting the ETS-PARP1 axis in prostate cancer and possibly other ETS gene fusion-dependent cancers. Although directly inhibiting transcription factors, such as ERG, may be difficult, blocking the function of regulatory cofactors, such as PARP1, is more feasible and may represent a viable treatment paradigm in cancer therapy.

In particular, PARP1 represents a very promising therapeutic target. Based on its role in base excision repair, PARP1 has been explored in both preclinical and clinical settings as a target in tumors with deficiencies in double-stranded DNA repair, such as mutations in BRCA1 and BRCA2 (Bryant et al., 2005; Farmer et al., 2005). In these cancers, the inhibition of PARP1 in cells with an inherent defect in homologous repair results in stalled replication forks and subsequent cell death (Bryant et al., 2005; Farmer et al., 2005). An initial phase I trial of the PARP inhibitor Olaparib has suggested an excellent therapeutic response in patients with BRCA1/2-deficient tumors from multiple organ sites with most patients experiencing a large reduction in total tumor volume (Fong et al., 2009). However, most cancers do not harbor BRCA1/2 mutations; only 5%–6% of all breast cancers are associated with an inherited BRCA1/2 gene mutation (Malone et al., 1998), and only 3% of prostate tumors from an Ashkenazi Jewish population of 832 patients were BRCA1/2 deficient (Gallagher et al., 2010).

Although PARP inhibitors can exploit the DNA-repair defects of BRCA-deficient tumors to induce cell death, we now demonstrate that they can also inhibit ETS gene fusion protein activity by preventing ETS transcriptional activity, inhibiting ETS-associated invasion, and enhancing ETS-mediated DNA damage. Future studies will help determine if, as with AR-mediated transcription (Haffner et al., 2010), ETS-mediated transcription is directly coupled to the induction of DNA damage. Importantly, the potentiation of ETS-induced DNA damage by PARP inhibition is of particular clinical interest, analogous to the "synthetic lethality" resulting from PARP inhibition in BRCA1/2-deficient tumors. By suggesting that cancers driven by specific oncogenic transcription factors may respond to PARP inhibition independent of BRCA1/2 status, our data support the notion that multiple tumor subtypes will be susceptible to PARP pharmacotherapy. It is important to note that the company Sanofi-BiPAR recently released a press report that their phase III trial assessing the addition of their next generation PARP inhibitor, Iniparib, to a gemcitabine-carboplatin regimen for patients with metastatic triple-negative breast cancer, was negative for an overall survival benefit (http://sanofi-aventis.mediaroom.com/index.php?s = 43 &item = 310). This is in direct contrast to the recently reported phase II trial showing that the addition of Iniparib approximately doubled overall survival in this setting (O'Shaughnessy et al., 2011), and some questions about specificity have been raised (Carey and Sharpless, 2011). Nonetheless, these results highlight the importance of target selection; it is expected that ongoing phase III trials assessing chemotherapy with or without PARP inhibitor in BRCA mutant cancers (instead of a nonspecific triple-negative breast cancer population) will be positive because the patient population is selected based on the presence of the PARP inhibitor target—BRCA mutation (Ellisen, 2011). Here, we have shown that Olaparib very specifically, and in a dose-dependent manner, delays tumor growth of ETS-positive, but not ETS-negative, prostate cancer xenografts.

By exploiting the ETS:PARP1 interaction to selectively target ETS-overexpressing xenografts, our studies significantly expand the total population of patients who could benefit from PARP inhibition. Consequently, the data presented here also have implications on the design of subsequent clinical trials that will follow the recently reported phase I trial of Olaparib (Fong et al., 2009). Although most trials will undoubtedly be designed to target and subtype BRCA-deficient tumors, trials

designed in organ sites that are also known to harbor aberrantly expressed ETS genes, such as breast, melanoma, Ewing's sarcoma, and especially prostate, should also subtype the disease by ETS status. Based on the data presented here, ETS-positive tumors are expected to respond with a higher probability to PARP inhibition than ETS-negative tumors, potentially making ETS status an important predictive biomarker. In line with the observation that PARP inhibitors can significantly increase the mean overall survival of patients with triple-negative breast cancer when added onto an existing regimen, our data suggest that the best design for a clinical trial in hormone-refractory metastatic prostate cancer will be to add PARP inhibitors in combination with chemotherapeutics known to potentiate the effects of PARP inhibition such as TMZ.

Finally, the observation that gene fusions that drive the gross overexpression of ETS genes also induce DNA double-strand break formation provides additional mechanistic insight into how ETS gene fusions drive cancer progression. Specifically, by causing DNA double-strand breaks, ETS gene overexpression may also play a role in the gradual evolution of genomic rearrangements. This finding may explain why recurrent ETS gene fusions were difficult to discover because ETS overexpression simply leads to the accumulation of additional gene fusions, only some of which will drive disease progression. In fact this model may partially explain the clinical behavior of prostate cancers that lie dormant for years only to spontaneously become extremely aggressive.

EXPERIMENTAL PROCEDURES

Xenograft and Primary Human Xenograft Models

For human prostate cancer xenografts, written informed consent was obtained before sample acquisition, and the sample was processed according to a protocol approved by The University of Texas, MD Anderson Cancer Center institutional review board. All procedures were approved by the University of Michigan's University Committee on Use and Care of Animals (UCUCA). Expression profiling was performed using the Agilent Whole Human Genome Oligo Microarray (Santa Clara, CA, USA) according to the manufacturer's protocol and described previously (Tomlins et al., 2007b).

ACCESSION NUMBERS

Coordinates have been deposited in the GEO with accession code GSE27723.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, six figures, and two tables and can be found with this article online at doi:10.1016/j.ccr.2011.04.010.

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